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## ORIGINAL ARTICLE

# CLEC-2-dependent activation of mouse platelets is weakly inhibited by cAMP but not by cGMP

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**Summary.** *Background:* The activation of platelet CLEC-2 by podoplanin on lymphatic endothelial cells (LECs) has a critical role in prevention of mixing of lymphatic and blood vasculatures during embryonic development. Paradoxically, LECs release cAMP and cGMP-elevating agents, prostacyclin (PGI<sub>2</sub>) and nitric oxide (NO), respectively, which are powerful inhibitors of platelet activation. This raises the question of how podoplanin is able to activate CLEC-2 in the presence of the inhibitory cyclic nucleotides. *Objectives:* We investigated the influence of cyclic nucleotides on CLEC-2 signaling in platelets. *Methods:* We used rhodocytin, CLEC-2 monoclonal antibody, LECs and recombinant podoplanin as CLEC-2 agonists on mouse platelets. The effects of the cyclic nucleotide-elevating agents PGI<sub>2</sub>, forskolin and the NO-donor GSNO were assessed with light transmission aggregometry, flow cytometry, protein phosphorylation and fluorescent imaging of platelets on LECs. *Results:* We show that platelet aggregation induced by CLEC-2 agonists is resistant to GSNO but inhibited by PGI<sub>2</sub>. The effect of PGI<sub>2</sub> is mediated through decreased phosphorylation of CLEC-2, Syk and PLCγ2. In contrast, adhesion and spreading of platelets on recombinant podoplanin, CLEC-2 antibody and LECs is not affected by PGI<sub>2</sub> and GSNO. Consistent with this, CLEC-2 activation of Rac, which is required for platelet spreading, is not altered in the presence of PGI<sub>2</sub>. *Conclusions:* The present results demonstrate that platelet adhesion and activation on CLEC-2

ligands or LECs is maintained in the presence of PGI<sub>2</sub> and NO.

**Keywords:** blood platelets; C-type lectin; cyclic nucleotides; lymphangiogenesis; platelet activation.

The C-type lectin receptor CLEC-2 has a single YxxL (hemITAM) in its cytoplasmic tail, which is phosphorylated upon ligand engagement by the interplay of Src and Syk tyrosine kinases [1]. In turn, Syk is recruited via its tandem SH2 domains to two phosphorylated CLEC-2 tails, leading to initiation of a downstream signaling cascade involving LAT, SLP-76, PI3 kinase and PLCγ2, which culminates in platelet activation [2].

The only established physiological ligand for CLEC-2 is podoplanin, a sialomucin-like glycoprotein expressed in a variety of cells, including lymphatic endothelial cells (LECs). Several groups have shown that deletion of the gene encoding CLEC-2, *Clec1b*, results in perinatal lethality in association with defects in lymphatic development [3–5] and a failure to inflate the lungs at birth [5]. PF4-Cre *Clec1b*<sup>fl/fl</sup> transgenic mice, which are deficient of CLEC-2 in the platelet/megakaryocyte lineage, also have blood-filled lymphatics [5,6]. The same phenotype is seen following deletion of the CLEC-2 signaling proteins, Syk, SLP-76 and PLCγ2 [4,5,7], or its ligand, podoplanin [4]. These observations indicate that the activation of CLEC-2 on platelets by podoplanin is necessary for prevention of blood-lymphatic mixing during embryonic development [5,6]. Blood-filled lymphatics are found in the intestines of radiation chimeric mice reconstituted with *Clec1b*<sup>-/-</sup> fetal liver cells, indicating that CLEC-2 is also necessary to repair the integrity of the intestinal lymphatic system. The interaction between platelet CLEC-2 and podoplanin expressed on reticular fibroblastic cells is important in high endothelial venules, where it maintains integrity during immune responses [8].

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The cyclic nucleotide-elevating agents, NO and PGI<sub>2</sub>, are released by endothelial cells in the vasculature [9]. LECs generate prostanoids, including PGI<sub>2</sub> [10,11], as well as NO [12], and these have been shown to modulate the contractile activity of the collecting lymphatics [13,14]. The observation that podoplanin and CLEC-2 are critical for prevention of blood-lymphatic mixing during development raises the question of how podoplanin is able to activate CLEC-2 in the presence of PGI<sub>2</sub> and NO, and therefore elevated cAMP and cGMP, given the powerful inhibitory action of the two cyclic nucleotides [9,15].

Herein, we show that platelet aggregation induced by rhodocytin, CLEC-2 antibody or podoplanin is inhibited by PGI<sub>2</sub> but not by NO-donors, and that platelet spreading on CLEC-2 agonists is relatively insensitive to cyclic nucleotide-elevating agents. These observations have important implications for understanding the molecular basis of platelet regulation in the development of the lymphatic system in mice.

## Experimental procedures

### Materials

Rhodocytin was purified as previously described [16]. The extracellular domain (ECD) of mouse podoplanin was amplified from cDNA generated from C57BL/6 kidney with the primers mPodoHindFor (GATCAAGCTTATG TGGACCGTGCCAGTGTTG) and mPodoFcRev (GAT CGGATCCACTTACCTGTCAGGGTGACTACTGGCA AGCC). After digestion with HindIII and BamHI the PCR product was cloned into a human IgG-Fc containing vector. Recombinant protein was expressed and purified as previously described [17]. Thrombin, PGI<sub>2</sub>, forskolin, GSNO and formaldehyde were purchased from Sigma (Poole, UK). Chrono lume and ATP were obtained from Chrono Log Corporation (Manchester, UK). PRT060318 was from previously described sources [1]. Fibrinogen was from Enzyme Research (Swansea, UK), and Hydromount from National Diagnostic (Atlanta, GA, USA). Alexa-Fluor-488-labeled fibrinogen was from Molecular Probes (Paisley, UK). Primary human LECs of dermal origin (HLECs) were purchased from Promocell GmbH (Heidelberg, Germany), and  $\mu$ -slides and Ibidi mounting medium were from Ibidi GmbH (Martinsried, Germany). ECL reagents were from GE Healthcare (Little Chalfont, UK). Lotrafiban was from GlaxoSmithKline (Philadelphia, PA, USA). Leakage resistant Fura-2 AM was from Teflabs (Austin, Texas, USA). The Rac1 activity kit was from Millipore (Billerica, MA, USA). The antibodies used in this study are listed in Table S1.

### Mouse platelet preparation

Wild-type (C57Bl/6 background) mice were anesthetized and terminally CO<sub>2</sub> asphyxiated prior to laparotomy.

Blood was drawn into 10% ACD (85 mM sodium citrate, 111 mM glucose, 78 mM citric acid). Washed platelets were prepared and resuspended in Tyrode's buffer as previously described [18]. Washed platelets were incubated at room temperature (RT) for at least 30 min prior to experimentation to allow the effects of PGI<sub>2</sub> to decay.

### HLECs culture

HLECs were cultured in adherence on 1% gelatin in endothelial cell growth medium according to the manufacturer's instructions. Prior to aggregation experiments, cells were non-enzymatically detached, suspended in Tyrode's buffer and immediately used. For adhesion experiments cells were cultured in Ibidi  $\mu$ -slides to confluence for 24 h prior to use. Cells were used between passages 4 and 7.

### Platelet aggregation and dense granule secretion

Aggregation was performed in a dual channel Born lumi-aggregometer (Chronolog, Labmedics, Manchester, UK) at 37 °C with stirring at 1200 rpm. When stated, washed platelets ( $2 \times 10^8$  per mL) were incubated with apyrase  $2 \text{ U mL}^{-1}$  and indomethacin  $10 \mu\text{M}$  for 15 min prior to agonist addition. PGI<sub>2</sub> or GSNO was pre-incubated with platelets for 3 min at 37 °C. ATP secretion was measured by luminometry at the same time using Chronolume 1 : 30 and calibrating with a known amount of ATP standard.

### Platelet spreading

Glass coverslips were coated overnight at 4 °C with  $10 \mu\text{g mL}^{-1}$  mouse podoplanin, CLEC-2 mAb or  $100 \mu\text{g mL}^{-1}$  fibrinogen and blocked with  $5 \text{ mg mL}^{-1}$  denatured BSA for 1 h at RT. Washed platelets ( $2 \times 10^7$  per mL) treated as indicated were allowed to spread on the coverslips for 45 min at 37 °C. Coverslips were washed, fixed with 4% paraformaldehyde and mounted in Hydromount. Imaging was performed using differential interference contrast (DIC) optics with a Zeiss 63 $\times$  oil immersion 1.40 NA plan-apochromat lens on a Zeiss Axiovert 200M microscope (Cambridge, UK). Images were captured by a Hamamatsu Orca 285 cooled digital camera (Welwyn Garden City, UK). The surface area of spread platelets was quantified by manual outlining and adhesion was quantified by counting platelets/field in five fields per experiment. The number of pixels within each outline was determined using Image J software. For adhesion on HLECs, platelets ( $5 \times 10^8$  per mL), treated as stated, were allowed to spread in HLEC-coated Ibidi  $\mu$ -slides for 1 h at 37 °C prior to fixation and immunostaining with PE-conjugated anti-human podoplanin antibody (NZ-1.3), rat anti-mouse CD41 antibody (MWReg30) and goat anti-rat IgG-Alexa 647, mouse anti-CD62P antibody (Psel.KO.2.7) and goat anti-mouse IgG-Alexa 488. Slides were mounted in Ibidi mounting

medium and imaged with a 63 $\times$  oil immersion 1.40 NA objective in a Leica SP2 confocal microscope (Wetzlar, Germany). Image analysis was performed using Image J software after applying automatic thresholds to segment raw greyscale images into binary data.

#### *Ca<sup>2+</sup> imaging*

Platelets ( $2 \times 10^8$  per mL) were labeled with 2  $\mu$ M leakage resistant Fura-2 AM for 1 h, spun and suspended at  $2 \times 10^7$  per mL. Aliquots treated as stated were added to a monolayer of HLECs in MatTek glass bottom dishes and imaged with 100 $\times$  magnification (Olympus IX71 microscope [Southend on Sea, UK] coupled with a Photometrics Evolve 512 EMCCD Camera [Tucson, AZ, USA]) for 20 min every 2.5 s. Excitation wavelengths were alternated between 340 and 380 nm and emission was captured at 510 nm. Emission ratios for 340/380 excitations were processed using Metafluor software (Universal Imaging Corp., Downingtown, PA, USA). Calibration of the fluorescent dye maximum and minimum for the optical set-up was done in situ and  $[Ca^{2+}]_i$  was calculated according to Grynkiewicz [19].

#### *Immunoprecipitation*

Stimulations for the preparation of lysates were performed as reported [1]. Washed platelets ( $4 \times 10^8$  per mL) were incubated with 10  $\mu$ M lotrafiban for 15 min to prevent aggregation. When stated, apyrase 2 U mL<sup>-1</sup>, indomethacin 10  $\mu$ M, PGI<sub>2</sub> 1–2  $\mu$ M or GSNO 100  $\mu$ M were incubated as described above. Stimulations were stopped after 3 min by adding 2 $\times$  lysis buffer containing protease and phosphatase inhibitors. Immunoprecipitations were performed as reported. Modifications from standard protocols were made for mouse CLEC-2 immunoprecipitations as previously described [1].

#### *SDS-PAGE and western blotting*

SDS-PAGE (10%) and western blot were performed as described [1]. When necessary, antibodies were removed by incubation with stripping buffer (TBS-T containing 2% SDS) supplemented with 1%  $\beta$ -mercaptoethanol for 20 min at 80 °C followed by incubation with stripping buffer only. Densitometry of the bands was performed using Adobe Photoshop.

#### *Data analysis*

Data are shown from a single representative experiment or as arithmetic mean  $\pm$  SEM from the stated number of experiments. Statistical analysis was carried out using one-way or two-way ANOVA followed by the stated post-test.  $P < 0.05$  was considered significant.

## **Results**

### *Role of secondary mediators in CLEC-2-dependent mouse platelet activation*

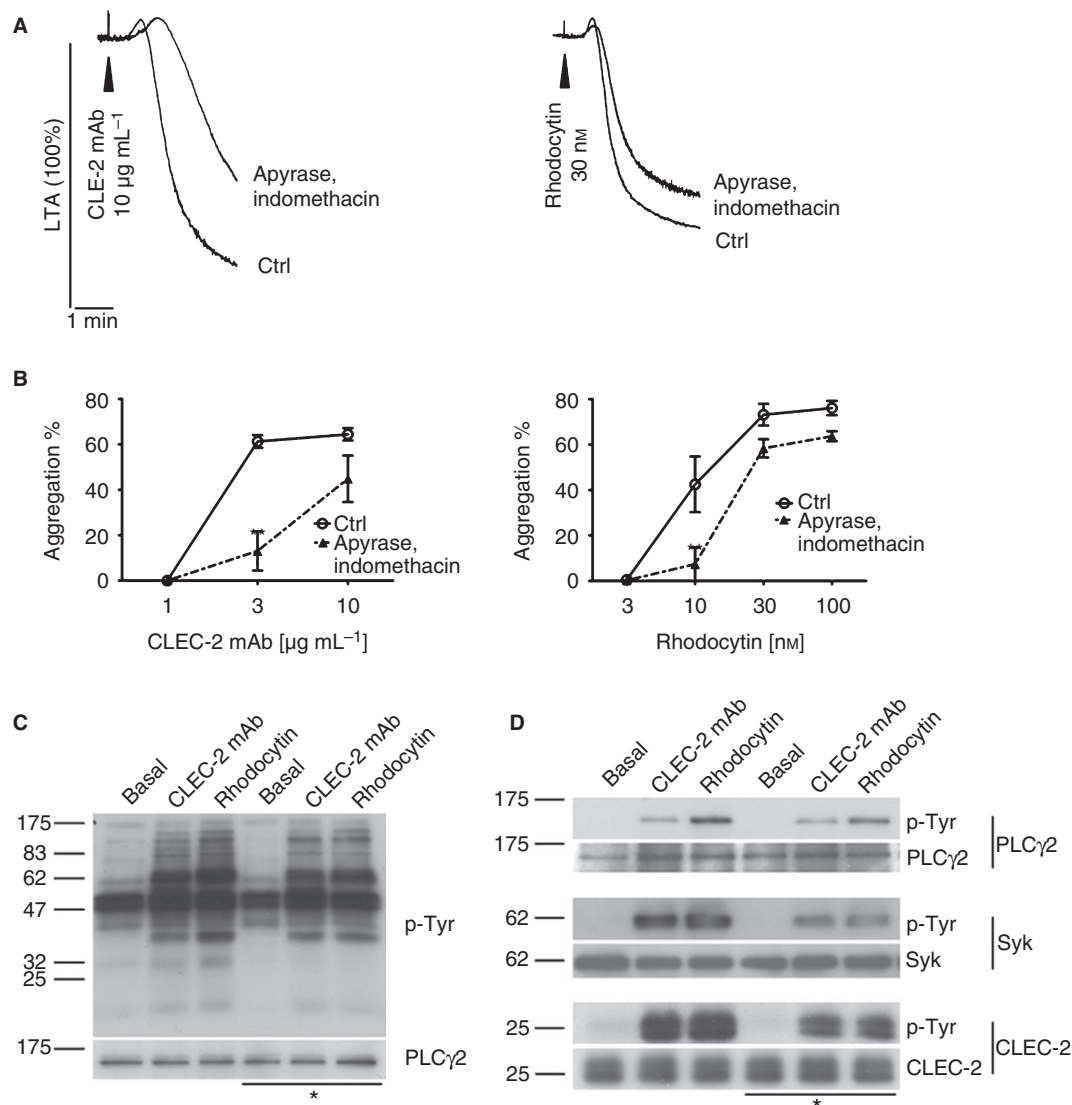
We have shown that CLEC-2-dependent activation of human platelets is regulated by release of the secondary mediators ADP and TXA<sub>2</sub> [20]. To investigate the role of the two feedback agonists in mouse platelets, we used apyrase (2 U mL<sup>-1</sup>) and indomethacin (10  $\mu$ M) to remove ADP and prevent TXA<sub>2</sub> synthesis, respectively. On their own, apyrase and indomethacin had no significant effect on aggregation induced by rhodocytin, and caused a minor delay in response to 3  $\mu$ g mL<sup>-1</sup> CLEC-2 mAb (Figure S1). These inhibitors, however, acted synergistically, causing a delay in aggregation in response to high concentrations of the CLEC-2 mAb and rhodocytin, and a significant reduction at low and intermediate concentrations (Fig. 1A,B).

The role of secondary mediators in protein tyrosine phosphorylation was investigated. Lotrafiban was used to block aggregation and outside-in signaling from integrin  $\alpha$ IIb $\beta$ 3. Global tyrosine phosphorylation of platelet proteins and phosphorylation of CLEC-2, Syk and PLC $\gamma$ 2 were studied in response to 10  $\mu$ g mL<sup>-1</sup> CLEC-2 mAb or 30 nM rhodocytin (Fig. 1C,D). For both agonists, phosphorylation was partially reduced in the presence of apyrase and indomethacin, as was particularly evident in the immunoprecipitation studies.

These results demonstrate that activation of mouse platelets by CLEC-2 is potentiated by the secondary agonists, ADP and TxA<sub>2</sub>, although to a lesser extent than for human platelets [20].

### *Effects of cyclic nucleotides on CLEC-2-dependent platelet aggregation*

We used PGI<sub>2</sub> and the NO-donor GSNO to elevate intracellular cAMP and cGMP, respectively. Dose response curves for PGI<sub>2</sub> and GSNO against concentrations of agonists that induce maximal aggregation (10  $\mu$ g mL<sup>-1</sup> CLEC-2 mAb, 30 nM rhodocytin) are shown (Fig. 2A,B). CLEC-2 mAb and rhodocytin-induced platelet aggregation were completely abolished by 2  $\mu$ M PGI<sub>2</sub>. In contrast, GSNO, at concentrations up to 1 mM, had no significant effect on aggregation in response to either agonist. ATP secretion from dense granules following 10  $\mu$ g mL<sup>-1</sup> CLEC-2 mAb activation was completely abolished by 1  $\mu$ M PGI<sub>2</sub> and unchanged with 1 mM GSNO ( $3.5 \pm 1.0$  vs.  $3.6 \pm 0.1$  nmol). Partial inhibition of aggregation was achieved against a threshold concentration of 3  $\mu$ g mL<sup>-1</sup> CLEC-2 mAb ( $28.4 \pm 10.6$  vs.  $59.6 \pm 5.1\%$ ;  $P < 0.05$ ) but not against rhodocytin (Fig. 2Ci–ii). A similar set of results was observed with a second NO-donor (sodium nitroprusside, SNP, Figure



**Fig. 1.** CLEC-2-dependent activation of mouse platelets is partially mediated by ADP and  $\text{TxA}_2$ . Washed platelets ( $2 \times 10^8$  per mL) were stimulated with  $10 \mu\text{g mL}^{-1}$  CLEC-2 mAb or 30 nM rhodocytin in the presence or absence of  $2 \text{ U mL}^{-1}$  apyrase and  $10 \mu\text{M}$  indomethacin. Representative light transmission aggregometry (LTA) traces are shown (A). Aggregation after 5 min was plotted as mean  $\pm$  SEM ( $n = 4-6$ ). Statistical differences were evaluated by two-way ANOVA and Bonferroni post-test ( $**P < 0.01$ ) (B). Washed platelets ( $4 \times 10^8$  per mL) incubated with  $2 \text{ U mL}^{-1}$  lotrafiban were stimulated with  $10 \mu\text{g mL}^{-1}$  CLEC-2 mAb and 30 nM rhodocytin for 3 min in the presence (\*) and absence of  $2 \text{ U mL}^{-1}$  apyrase and  $10 \mu\text{M}$  indomethacin. Whole cell lysate was analyzed by SDS-PAGE (C) and immunoprecipitation of PLC $\gamma$ 2, Syk and CLEC-2 (D). Blots were probed with anti phospho-tyrosine (p-Tyr) antibody and reprobed for equal loading control ( $n \geq 3$ ) (C&D).

S2A). In contrast, GSNO  $100 \mu\text{M}$  causes a marked inhibition of thrombin-induced aggregation (Fig. 2D).

As recombinant podoplanin and podoplanin-expressing cells are able to induce platelet aggregation [21,22], HLECs ( $2 \times 10^6$ ) were used as a physiological CLEC-2 agonist as they express podoplanin. HLECs induced platelet aggregation after a lag phase of several minutes, which was abolished by  $\text{PGI}_2$  ( $2 \mu\text{M}$ ) but not affected by GSNO ( $1 \text{ mM}$ ) (Fig. 2E).

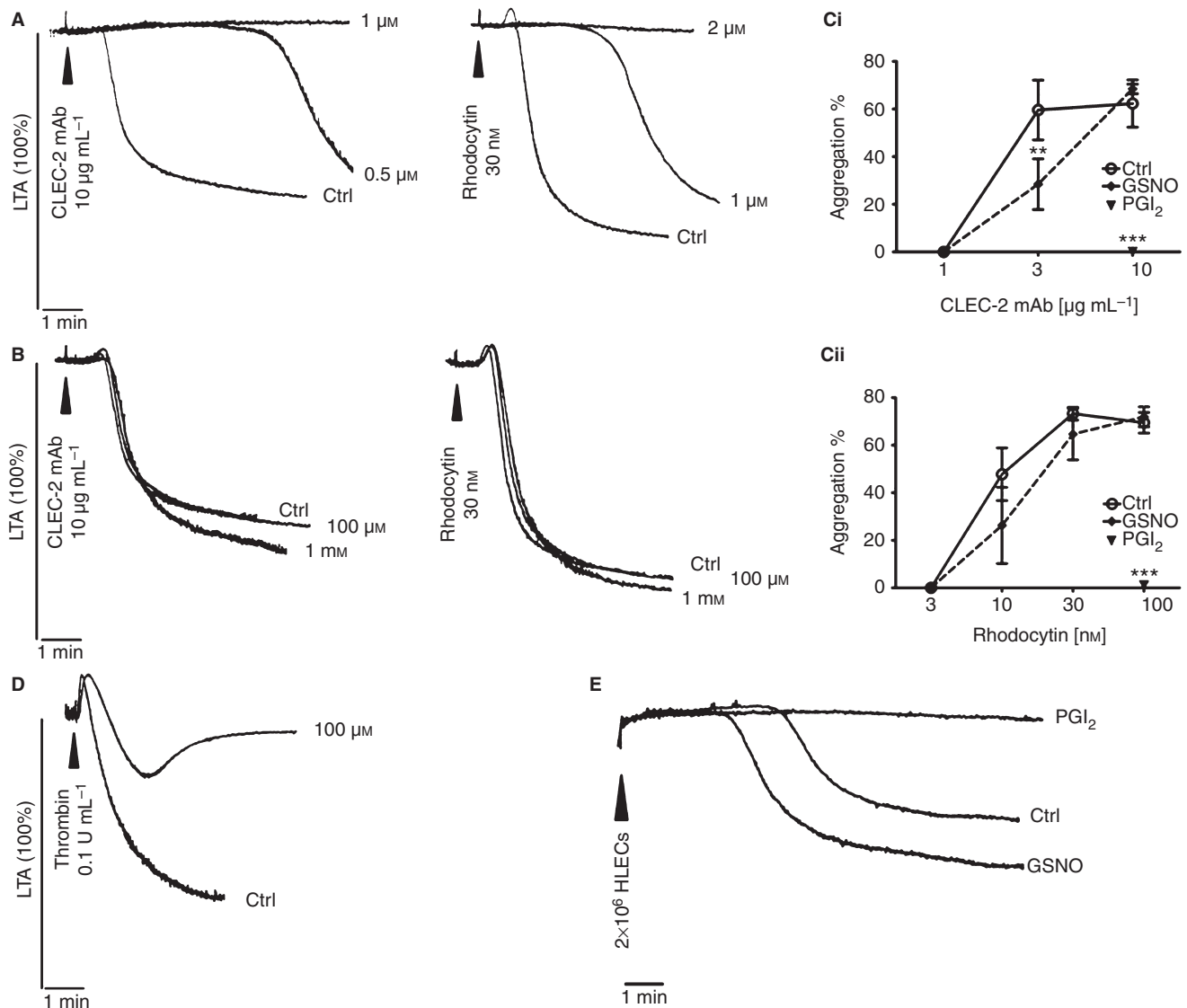
These results demonstrate that elevation of cAMP but not cGMP inhibits platelet aggregation to CLEC-2 agonists.

A similar result was observed in human platelets activated by rhodocytin (Figure S3A), although a lower concentration of  $\text{PGI}_2$  was sufficient to inhibit aggregation as compared with mice. In contrast, neither GSNO nor SNP inhibited aggregation at concentrations of up to  $300 \mu\text{M}$  (not shown).

#### Effects of cyclic nucleotides on CLEC-2-dependent tyrosine phosphorylation

CLEC-2 mAb stimulates marked tyrosine phosphorylation of cellular proteins in the presence of inhibitors of





**Fig. 2.** CLEC-2-dependent platelet aggregation is sensitive to cAMP-mediated inhibition. Washed platelets ( $2 \times 10^8$  per mL) were stimulated with CLEC-2 mAb or rhodocytin in the presence of increasing concentrations of (A)  $\text{PGI}_2$  or (B) GSNO. Representative aggregation traces are shown. Aggregation at 5 min in the presence or absence of either  $\text{PGI}_2$  ( $1 \mu\text{M}$  for CLEC-2 mAb,  $2 \mu\text{M}$  for rhodocytin) or GSNO ( $1 \text{ mM}$ ) was plotted as mean  $\pm$  SEM ( $n = 3-5$ ). Statistical differences were evaluated by two-way ANOVA and Bonferroni post-test (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ ) (C). Washed platelets were stimulated with  $0.1 \text{ U mL}^{-1}$  thrombin in the presence of  $100 \mu\text{M}$  GSNO; representative traces are shown (D). Washed platelets were stimulated with HLECs ( $2 \times 10^6$ ) in the presence or absence of  $2 \mu\text{M}$   $\text{PGI}_2$  or  $1 \text{ mM}$  GSNO (E) ( $n = 3$ ).

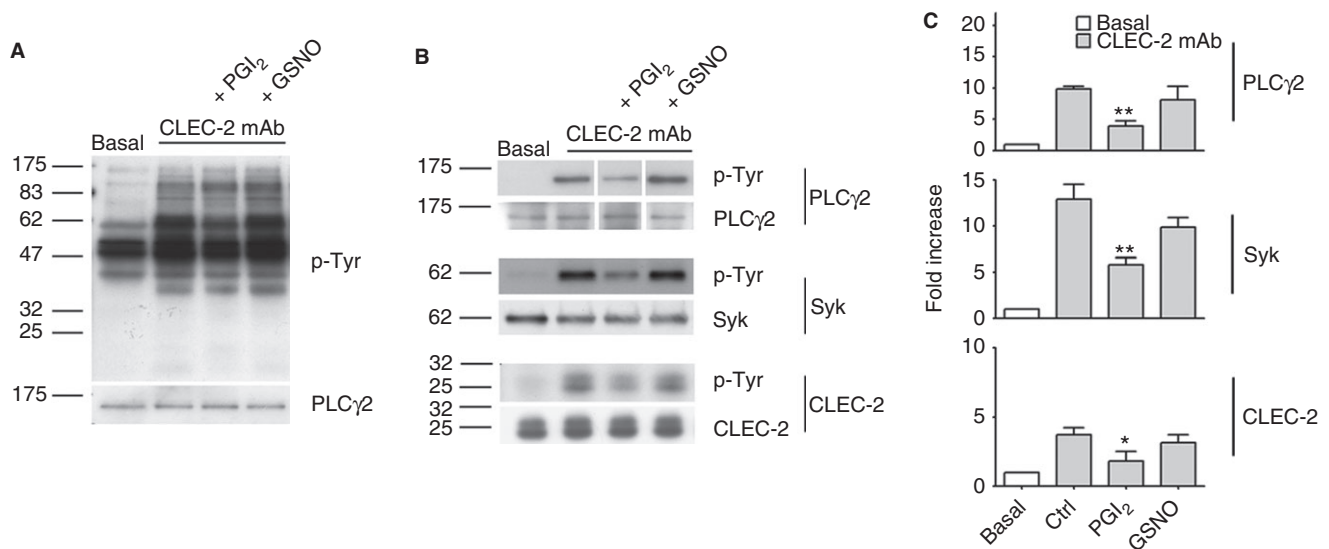
secondary mediators, which was reduced in the presence of  $1 \mu\text{M}$   $\text{PGI}_2$  but unaltered in the presence of  $100 \mu\text{M}$  GSNO (Fig. 3A) or SNP (Figure S2B).  $\text{PGI}_2$  inhibited phosphorylation of Syk and  $\text{PLC}\gamma 2$  by over 50% and of CLEC-2 to a lesser degree (Fig. 3B,C). Similar results were observed for rhodocytin (Figure S4A,B). Interestingly, CLEC-2 mAb and rhodocytin stimulated a similar level of phosphorylation of Syk and CLEC-2, while rhodocytin induced stronger  $\text{PLC}\gamma 2$  phosphorylation, possibly as a result of sustained receptor clustering [23].

In human platelets CLEC-2-dependent tyrosine phosphorylation strongly depends on secondary mediators

[20]. The residual signal in the presence of apyrase and indomethacin is very weak (Figure S3B) but was inhibited by  $\text{PGI}_2$  and not by cGMP-elevating agents, in line with the observations in mouse platelets.

#### Effect of cyclic nucleotides on CLEC-2-dependent spreading

The effect of cyclic nucleotides on adhesion and spreading of platelets to mouse podoplanin and CLEC-2 mAb was investigated. A concentration of  $\text{PGI}_2$  ( $1 \mu\text{M}$ ) that completely inhibited aggregation and secretion had no effect on spreading of platelets on either ligand (Fig. 4A,B). A



**Fig. 3.** CLEC-2-dependent tyrosine phosphorylation is partially reduced by cyclic nucleotide-elevation. Washed platelets ( $4 \times 10^8$  per mL) incubated with PGI<sub>2</sub> (1  $\mu$ M) or GSNO (100  $\mu$ M) in the presence of apyrase (2 U mL<sup>-1</sup>), indomethacin (10  $\mu$ M) and lotrafin (10  $\mu$ M) were stimulated with 10  $\mu$ g mL<sup>-1</sup> CLEC-2 mAb for 3 min and lysed. Aliquots were analyzed by SDS-PAGE (A) and immunoprecipitation of PLCγ2, Syk and CLEC-2 (B) ( $n = 3-4$ ). Blots were probed with anti phospho-tyrosine mAb (A & B) and reprobed for equal loading. Densitometry of PLCγ2, Syk and CLEC-2 bands was expressed as fold increase over basal and plotted as mean  $\pm$  SEM. Statistical differences were evaluated by one-way ANOVA test and Bonferroni post-test (\* $P < 0.05$ , \*\* $P < 0.01$ ) (C).

similar result was seen with a 10-fold higher concentration of PGI<sub>2</sub> (not shown). Similarly, GSNO (1 mM) had no effect. Weak inhibition of spreading was observed on CLEC-2 mAb with 10  $\mu$ M forskolin (Fig. 4A,B), which induces a sustained increase in cAMP. In contrast, spreading of thrombin-activated platelets on fibrinogen was markedly inhibited by PGI<sub>2</sub>, GSNO and forskolin (Fig. 4A,B). Platelet adhesion was unaltered under any of the above conditions (Fig. 4C).

Spreading of platelets on HLECs was less marked than on immobilized ligands, because of the mobility of podoplanin. Figure 5(A) shows exposure of P-selectin in CD41-stained platelets adhering to HLECs expressing podoplanin, thus confirming that platelet activation had occurred. Spreading (Fig. 5A,B), P-selectin exposure (Fig. 5C) and adhesion (not shown) on HLECs were unaltered or minimally reduced in the presence of PGI<sub>2</sub> (1  $\mu$ M), forskolin (10  $\mu$ M) or GSNO (1 mM), while mouse platelets expressing a functionally inactive Syk did not express P-selectin on HLECs (not shown). These results demonstrate that cAMP or cGMP elevation minimally inhibits or does not inhibit platelet activation by membrane-bound podoplanin. Consistent with this, neither cAMP nor cGMP inhibited the elevation of Ca<sup>2+</sup> in platelets on HLECs (Fig. 5D), whereas the Syk inhibitor PRT060318 (5  $\mu$ M) significantly reduced [Ca<sup>2+</sup>]<sub>i</sub>.

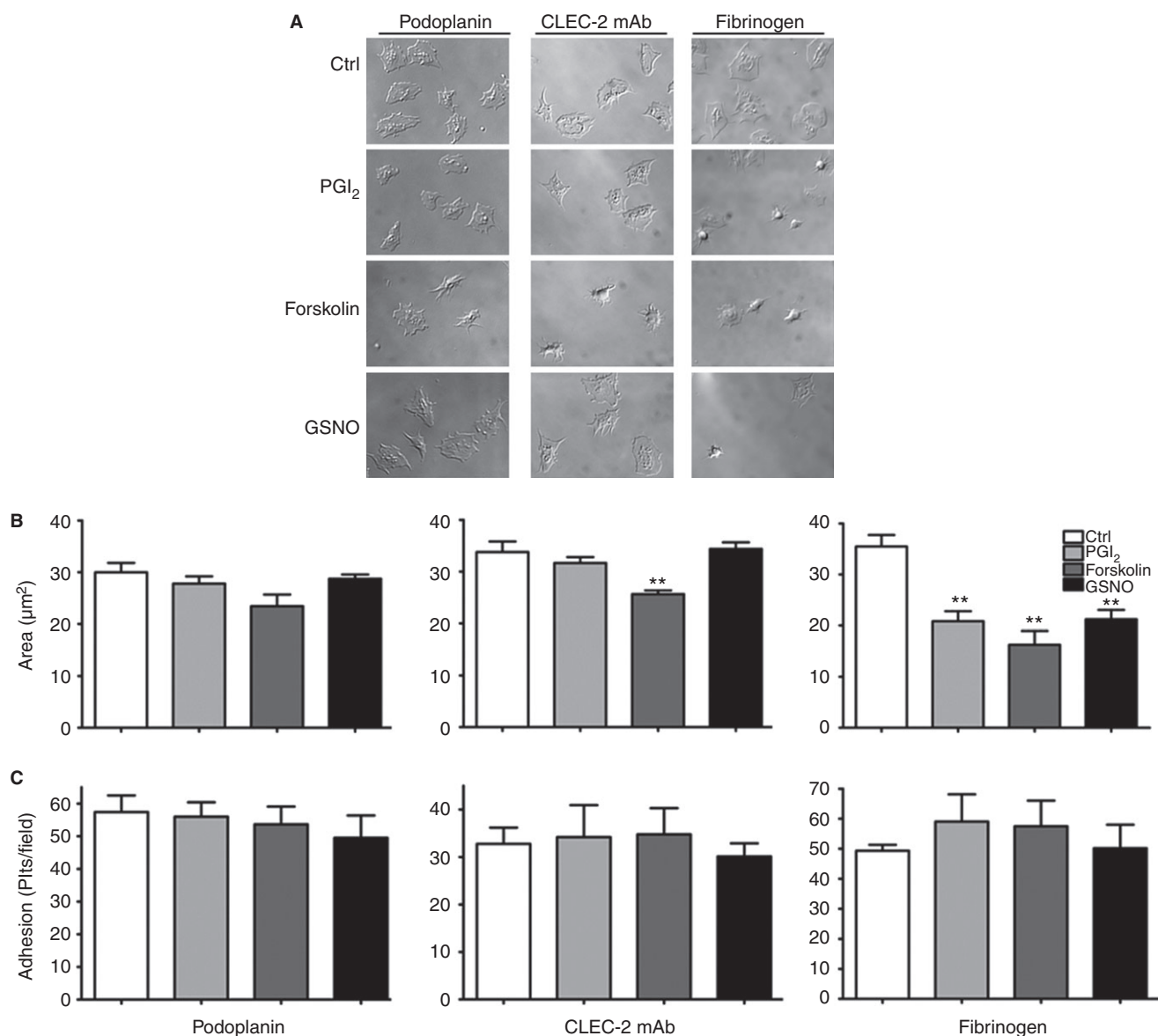
We have performed a series of experiments to establish the effectiveness of cyclic nucleotide-elevating agents at the different time-points used in this study (Figure S5). The activation of integrin  $\alpha$ IIb $\beta$ 3 measured by flow cytometry was inhibited by PGI<sub>2</sub> (or forskolin, not

shown) at 3 min and was retained by 45 min. In contrast, GSNO partially inhibited at 3 min but this effect was lost at 45 min, consistent with the aggregation results (Figure S5A,B).

The small G protein Rac has a critical role in platelet spreading on multiple surfaces [24]. Consistent with this, the Rac inhibitor EHT1864 blocked spreading on podoplanin (Figure S5C). Rac activation induced by CLEC-2 mAb in suspension was maintained in the presence of PGI<sub>2</sub> at 45 min, consistent with the absence of an effect on spreading at this time (Figure S5D). To confirm the effectiveness of PGI<sub>2</sub> and GSNO in activating PKA and PKG, respectively, in CLEC-2-stimulated platelets, we studied phosphorylation of the common PKA and PKG substrate VASPSer239 and PKA substrate VASPSer157 [15,25] (Figure S5E). PGI<sub>2</sub>-dependent VASP phosphorylation in Ser239 and 157 was lost after 3 min, consistent with the short life of the compound, even though inhibition of fibrinogen binding and fibrinogen spreading was maintained. Forskolin and GSNO-dependent VASPSer239 phosphorylation were maintained up to 60 min.

## Discussion

Cyclic nucleotide-elevating agents are released by intact endothelial cells from both vascular and lymphatic lineages and are powerful inhibitors of platelet activation. This therefore raises the question of how podoplanin on LECs is able to mediate platelet activation and thereby prevent blood-lymphatic mixing during development. The present study addresses this through demonstration that



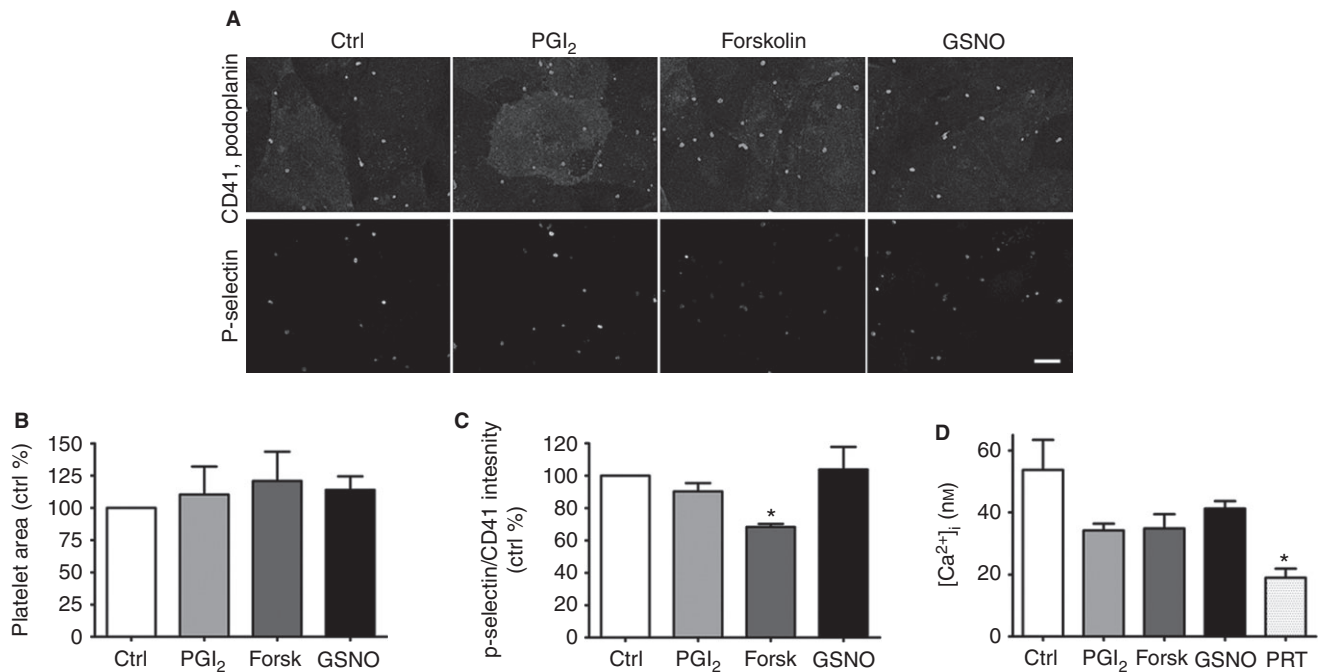
**Fig. 4.** cAMP-elevation marginally affects CLEC-2-dependent spreading.  $2 \times 10^7$  per mL washed platelets were treated with PGI<sub>2</sub> (1  $\mu$ M), forskolin (10  $\mu$ M) or GSNO (1 mM) before spreading on coverslips coated with 10  $\mu$ g mL<sup>-1</sup> recombinant podoplanin or CLEC-2 mAb. Control experiments with thrombin-activated platelets (0.1 U mL<sup>-1</sup>) on 100  $\mu$ g mL<sup>-1</sup> fibrinogen were performed. Coverslips were imaged using differential interference contrast (DIC) microscopy (A). Platelet areas are expressed as mean of mean area/experiment  $\pm$  SEM. One hundred platelets/experiment were analyzed. Statistical differences were evaluated by ANOVA and Dunnet's post-test (\*\* $P < 0.01$ ) (B). Results are expressed as mean of mean number of platelets/experiment  $\pm$  SEM ( $n = 3-6$ ) (C).

platelet activation by CLEC-2 ligands, including podoplanin, is only sensitive to elevation of cAMP in suspension, but not on an immobilized monolayer or on podoplanin-expressing cells. Furthermore, CLEC-2-dependent activation is resistant to elevation of cGMP both in suspension and on a cell surface. These observations are therefore consistent with the critical role of podoplanin-mediated platelet activation in prevention of blood-lymphatic mixing during development.

Our results allow us to draw a number of conclusions relative to the behaviour of cyclic nucleotides in mouse platelets and their effect on CLEC-2 activation. PGI<sub>2</sub>

prevents platelet aggregation in response to CLEC-2 ligands and this is partly mediated through impaired signaling by the C-type lectin receptor as revealed by measurement of tyrosine phosphorylation. In comparison, GSNO has a much weaker effect on platelet aggregation induced by CLEC-2, which is overcome by high concentrations of agonists. The weak effect of GSNO is likely to be due to a lower level of activation of PKG relative to PKA, as illustrated by the relatively weak phosphorylation of VASPSer239. PGI<sub>2</sub> and GSNO have no effect on platelet adhesion and spreading on immobilized CLEC-2 ligands or LECs, thereby demonstrating that the initial





**Fig. 5.** Cyclic nucleotides do not affect platelet spreading, P-selectin exposure and  $Ca^{2+}$  elevation on HLECs. Washed platelets ( $5 \times 10^8$  per mL) were treated with 1 μM PGI<sub>2</sub>, 10 μM forskolin, 1 mM GSNO or vehicle and allowed to spread on a HLEC monolayer for 1 h at 37 °C. Slides were stained for podoplanin, CD41 and P-selectin and imaged using confocal microscopy (scale bar = 20 μm) (A). Area of attached platelets (B) and P-selectin/CD41 intensity (C) were evaluated ( $n = 3$ ).  $2 \times 10^7$  per mL Fura-2-loaded platelets were treated as above or with 5 μM PRT060318 (PRT) and allowed to attach on a HLEC monolayer. Attachment of platelets was recorded for 20 min. Average  $[Ca^{2+}]_i$  concentrations were calculated and reported as mean  $\pm$  SEM ( $n = 3$ ). Statistical differences were evaluated with ANOVA and Dunnett's post-test (\* $P < 0.05$ ) (D).

events that underlie adhesion and spreading are refractory to cyclic nucleotides. Furthermore, P-selectin exposure and  $Ca^{2+}$  elevation are also not inhibited whereas only a very high concentration of forskolin has a partial effect on spreading on CLEC-2 mAb and P-selectin exposure on platelets adhering on LECs.

Lymphatic development is normal in mice deficient in  $\alpha IIb\beta 3$  [26], suggesting that it is independent of platelet aggregation. Thus, the inhibitory effect of PGI<sub>2</sub> against CLEC-2-mediated aggregation would not interfere with the role of CLEC-2 in lymphatic development. The molecular mechanism underlying the role of podoplanin and CLEC-2 in lymphatic development remains unclear. Previously, we have demonstrated that direct contact with platelets impairs LEC migration and cell-cell interactions through a pathway that is dependent on CLEC-2 and Syk [5]. The present results suggest that platelet adhesion to LECs and subsequent activation would not be altered by elevation of cGMP and only minimally impaired by elevation of cAMP. The present observations may have important implications with respect to the interaction of CLEC-2 and podoplanin on other cells. It has been proposed that the interaction of platelet CLEC-2 with podoplanin on circulating tumor cells plays a role in hematogenous metastatic dissemination [27]. In this case, platelets would be exposed to cyclic nucleotide-elevating agents produced by the intact

vascular endothelium, but these would have only a minimal effect on responses mediated by CLEC-2 signaling. This is consistent with the report that formation of human platelet aggregates on tumor cells under flow conditions is delayed but not abolished in the presence of PGI<sub>2</sub> [28].

In conclusion, we have demonstrated that CLEC-2-dependent platelet activation is insensitive to cGMP-elevation and only partially sensitive to cAMP-elevation, but only in suspension studies. The relative refractoriness of CLEC-2-dependent signaling to cyclic nucleotides when activated on a surface explains the ability of podoplanin-expressing LECs to maintain platelet activation, which is essential to prevent blood-lymphatic mixing during development.

### Addendum

A. Borgognone designed and performed experiments, analyzed data and wrote the manuscript. L. Navarro-Nunez and J. N. Correia designed and performed experiments and analyzed data. S. Thomas designed and performed experiments. A. Pollitt and J. Eble provided reagents and revised the manuscript. F. M. Pulcinelli and M. Madhani designed experiments and revised the manuscript. S. P. Watson designed experiments, wrote the manuscript and supervised the work.

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## Disclosure of Conflict of Interests

The authors state that they have no conflict of interests.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Antibodies used and sources.

**Fig. S1.** Marginal effect of apyrase or indomethacin on CLEC-2-dependent platelet aggregation.

**Fig. S2.** Lack of effect of SNP on CLEC-2-dependent platelet aggregation and tyrosine phosphorylation.

**Fig. S3.** Effect of cyclic nucleotide-elevation on CLEC-2-dependent platelet aggregation and tyrosine phosphorylation in human platelets.

**Fig. S4.** Rhodocytin-dependent tyrosine phosphorylation is partially reduced by cyclic nucleotide-elevation.

**Fig. S5.** cAMP and cGMP have differential effects on different platelet pathways.

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